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Biochemical Pharmacology 68 (2004) 1081–1087
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Eupatilin, a pharmacologically active flavone derived from *Artemisia* plants, induces cell cycle arrest in *ras*-transformed human mammary epithelial cells

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Received 2 March 2004; accepted 14 April 2004

Abstract

Extracts of *Artemisia asiatica* Nakai (Asteraceae) possess anti-inflammatory and anti-oxidative activities. Eupatilin (5,7-dihydroxy-3′,4′,6-trimethoxyflavone), one of the pharmacologically active ingredients derived from *A. asiatica*, was shown to induce apoptosis in human promyelocytic leukemia (HL-60) cells [Mutat Res 496 (2001) 191]. In the present study, we examined the cytostatic effects of eupatilin in H-*ras*-transformed human breast epithelial (MCF10A-*ras*) cells. Eupatilin inhibited the growth of MCF10A-*ras* cells in a concentration-dependent and time-related manner. To explore whether the anti-proliferative effects of eupatilin could be mediated through modulation of the cell cycle in MCF10A-*ras*, DNA contents were analyzed by the flow cytometry. Eupatilin inhibited the expression of cyclin D1, cyclin B1, Cdk2 and Cdc2 that are key regulators of the cell cycle. In addition, eupatilin treatment led to elevated expression of p53 and $p27^{Kip1}$ that act as Cdk inhibitors. It has been known that the Ras-signaling pathway plays integral roles in the induction of cyclin D1. Eupatilin inhibited the activation of ERK1/2 as well as expression of Raf-1 and Ras in MCF10A-*ras* cells. Thus, the inhibitory effect of eupatilin on cyclin D1 expression appears to be mediated by targeting the Raf/MEK/ERK signaling cascades. Eupatilin did not change activation of Akt, an important component of cell-survival pathways. In conclusion, the anti-proliferative effect of eupatilin in MCF10A-*ras* cells is associated with its blockade of cell cycle progression which appears to be attributable in part to inhibition of ERK1/2 activation.

Keywords: Eupatilin; Cell cycle arrest; MCF10A-*ras* cells; Ras/Raf/MEK; Cyclin D1

1. Introduction

One of the conventional strategies utilized in cancer chemoprevention/chemotherapy is preventing DNA synthesis or mitosis by blocking cell cycle progression in preneoplastic or malignant cells. Certain phytochemicals present in medicinal herbs and dietary plants exert a cytostatic effect by arresting growth of malignant or transformed cells.

Flavonoids are polyphenols ubiquitously found in a wide variety of edible plants. Many flavonoids exert potent anti-tumor activity through induction of apoptosis and cell cycle arrest in several cancer cell lines [1–3]. For example, a Citrus flavone tangeretin induced apoptosis in human promyelocytic leukemia (HL-60) cells [3] and blocked cell cycle progression at the G1 phase in human colorectal carcinoma COLO 205 cells [2].

Extracts of *Artemisia asiatica* Nakai (Asteraceae) possess anti-inflammatory and anti-tumor promoting activities [4]. Our previous studies have shown that eupatilin (5,7-dihydroxy-3′,4′,6-trimethoxy-flavone, structure shown in Fig. 1), one of the pharmacologically active ingredients of *A. asiatica*, induces apoptosis in HL-60 cells [5].

An uncontrolled Ras-signaling has been implicated in the development of human cancer [6,7]. The Ras-signaling pathway plays an important role in the regulation of cell proliferation by growth factors [8–11]. The aberrant activation of the mitogen-activated protein kinase (MAPK) pathway often results in the development, growth or spread of tumors [12,13]. Phosphatidylinositol 3-kinase (PI3K) and Akt/PKB play a crucial role in cell cycle control by targeting cyclin D1 which is regulated via the

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doi:10.1016/j.bcp.2004.04.027

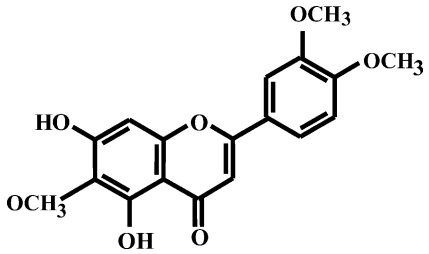


Fig. 1. Structural formula of eupatilin (5,7-dihydroxy-3′,4′,6-trimethoxy-flavone).

Ras-dependent signaling. While the Ras pathway that consists of Raf, MAPK kinase (MEK) and extracellular signal-regulated kinase (ERK) induces cyclin D1 synthesis, the PI3K/Akt/GSK-3β pathway is considered to selectively affect cyclin D1 stability [14,15].

In the present study, we evaluated the anti-tumorigenic potential of eupatilin by examining its capability to inhibit growth of H-*ras* transformed human breast epithelial (MCF10A-*ras*) cells. Here, we report that eupatilin exerts growth inhibitory effects in MCF10A-*ras* cells through modulation of key cell cycle regulators.

2. Materials and methods

2.1. Materials

Eupatilin was supplied from Dong-A Pharmaceutical Co. Ltd. (Yong-In, South Korea) and dissolved in DMSO for treatment. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], cholera toxin, hydrocortisone, insulin and h-EGF were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12 (1:1) and horse serum were obtained from Gibco BRL (Grand Island, NY, USA). The MEK inhibitor U0126 was obtained from Tocris Cookson Ltd. (Avonmouth, UK).

2.2. Cell culture

Cells were cultured in DMEM/F-12 medium supplemented with 5% heat-inactivated horse serum, 10 μg/ml insulin, 100 ng/ml cholera toxin, 0.5 μg/ml hydrocortisone, 20 ng/ml recombinant epidermal growth factor, 2 mmol/l L-glutamine and 100 units/ml penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

2.3. Determination of cell viability

MCF10A-*ras* cells were plated at a density of 5×10^4 cells in 48-well plates, and the cell viability was determined by the conventional MTT reduction assay. The MTT assay relies primarily on the mitochondrial metabolic capacity of viable cells and hence reflects the intracellular redox state. After incubation, cells were treated with the MTT solution (final concentration, 0.25 mg/ml) for 2 h at 37 °C. The dark blue formazan crystals formed in intact cells were dissolved with DMSO, and the absorbance was measured at 570 nm with an ELISA reader. Results were expressed as the percentage of MTT reduction, assuming that the absorbance of control cells was 100%.

2.4. Determination of DNA synthesis

Cells growing in six-well plates were treated with [methyl-^{3}H]thymidine (sp. act., 54 Ci/mmol) in varying concentrations of eupatilin. After incubation at 37 °C for 48 h, the medium was removed. The cells were harvested by treatment with trypsin-EDTA at 37 °C, rinsed with phosphate-buffered saline (PBS) and 10% trichloroacetic acid, and treated with 0.5% SDS in 0.2N NaOH at 37 °C for 30 min. The extent of [^{3}H]thymidine incorporation was measured by liquid scintillation counting.

2.5. Flow cytometry

Treated cells were trypsinized and washed with cold PBS once, fixed with 70% ethanol and stored at −20 °C until use. The fixed cells were then washed with PBS and stained with 20 μg/ml of propidium iodide containing 10 μg/ml RNase A. The stained cells were incubated at room temperature for 30 min in the dark. The DNA contents of the cells (1×10^4 cells/experimental group) were analyzed by a FACS Calibur flow cytometry using the CellQuest analysis program (BD Biosciences, North Ryde, Australia).

2.6. Western blot analysis

MCF10A-*ras* cells were lysed in RIPA lysis buffer [150 mM NaCl, 0.5% Triton × 100, 50 mM Tris–HCl (pH 7.4), 25 mM NaF, 20 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM Na_3VO_4, protease inhibitor cocktail tablet] for 15 min on ice followed by centrifugation at 12,000 × *g* for 20 min. The protein concentration of the supernatant was measured by using the BCA reagents (Pierce, Rockfold, IL, USA). Protein (30 μg) was separated by running through 12% SDS-PAGE gel and transferred to the PVDF membrane (Gelman Laboratory, Ann Arbor, MI, USA). The blots were blocked with 5% non-fat dry milk-PBST buffer [PBS containing 0.1% Tween-20] for 1 h at room temperature. The membranes were incubated for 2 h at room temperature with 1:1000 dilution of one of the polyclonal antibodies of p53, p21, p27, Cdc2, Cdk2, cyclin D1, cyclin B1, Raf-1 and ERK (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or pan-Ras (CALBIOCHEM, Germany) and phospho-ERK (Santa Cruz Biotechnology)

monoclonal antibodies. Equal lane loading was assessed using actin (Sigma Chemical Co., St. Louis, MO, USA). The blots were rinsed three times with PBST buffer for 5 min each. Washed blots were incubated with 1:5000 dilution of the horseradish peroxidase conjugated-secondary antibody (Zymed Laboratories, San Francisco, CA, USA) for 1 h and washed again three times with PBST buffer. The transferred proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

3. Results

3.1. Eupatilin caused reduced viability and proliferation of MCF10A-ras cells

The effect of eupatilin on the cell viability was measured by the MTT reduction assay. When MCF10A-*ras* cells were treated with 100 μM eupatilin, the cell viability declined in a time-related manner (Fig. 2A). To determine whether the reduced cell viability was due to decreased cell proliferation, we measured the incorporation of [^{3}H]thymidine into nuclei in the presence of varying amounts of eupatilin. As illustrated in Fig. 2B, DNA synthesis was inhibited in a concentration-dependent manner in MCF10A-*ras* cells treated with eupatilin.

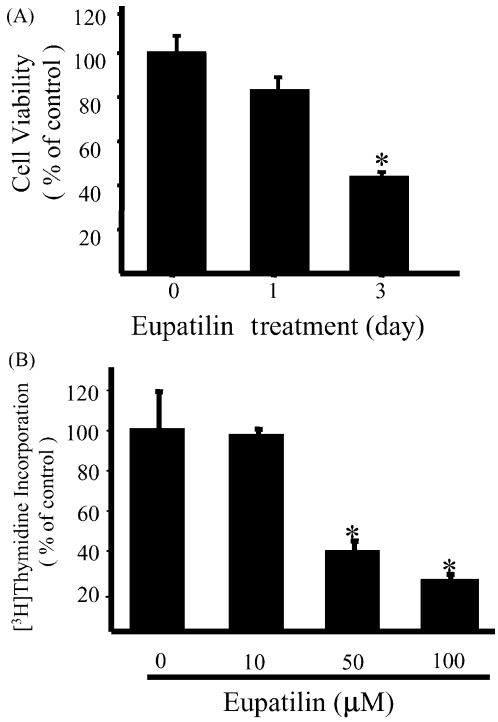
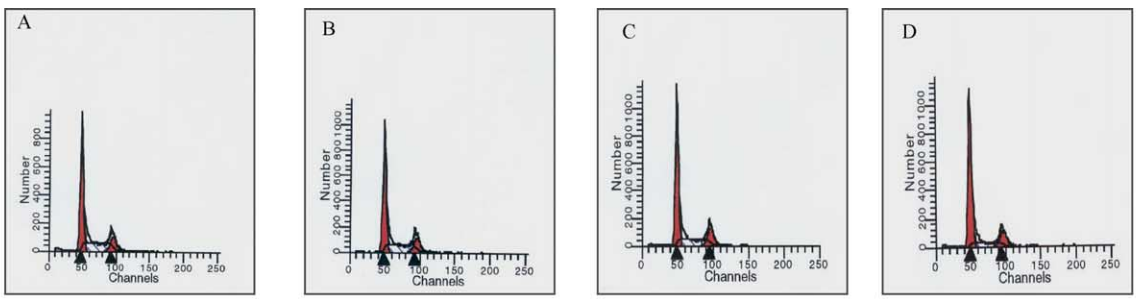


Fig. 2. (A) Effects of eupatilin on viability of MCF10A-*ras* cells. Cells were treated with 100 μM of eupatilin for indicated time periods, and the cell viability was determined by the MTT reduction assay. (B) Cell proliferation was assessed by measuring the incorporation of [^{3}H]thymidine into nuclei of cells after 2 day treatment with eupatilin. The results are presented as means ± S.D. ($n = 5$). Significantly different from untreated control: $^*P < 0.01$.



Phase / Cycle	Eupatilin (μM)			
	0	10	50	100
G1 (%)	58.77 ±2.37	60.85 ±2.15	64.74 ±2.01	70.20 ±1.27*
S (%)	29.24 ±2.47	25.28 ±2.02	20.09 ±1.54	15.61 ±1.20
G2/M (%)	12.01 ±0.31	13.87 ±0.20	15.18 ±0.66	14.19 ±0.20

Fig. 3. Effects of eupatilin on cell cycle distribution in MCF10A-*ras* cells. Cells were treated with 0 (A), 10 (B), 50 (C) or 100 μM (D) eupatilin for 48 h, and the cell cycle distribution was measured by flow cytometry analysis as described in Section 2. Data shown are representative of three independent experiments. Values are means ± S.D. ($n = 3$). *Significantly different from the untreated control ($P < 0.05$).

3.2. Eupatilin-induced cell cycle arrest at both G_1/S and G_2/M phases

Considering that eupatilin decreased cell proliferation, we analyzed its effect on cell cycle distribution by flow cytometry. Treatment with 100 μM eupatilin for 48 h caused accumulation of cells predominantly at the G_1 phase, with a concomitant decrease in the cell cycle progression through the S phase (Fig. 3). Eupatilin did not alter the sub-G_1 fraction, suggesting that it caused cell cycle arrest without induction of apoptosis.

3.3. Effect of eupatilin on the expression of cell cycle regulatory molecules: cyclins and Cdks

To elucidate the mechanisms involved in the cell cycle arrest caused by eupatilin, expression of the several major cell cycle regulators was assessed by Western blot analysis. Both cyclin D/Cdk4 and cyclin E/Cdk2 complexes are required for the cells to progress from G_1 into S phase. As shown in Fig. 4A, eupatilin (100 μM) caused reduced expression of cyclin D1 and Cdk2 at 48 h without altering the level of Cdk4. In addition, we examined the changes in the levels of proteins involved in controlling progression from G_2 to M phase. Eupatilin inhibited the expression of the G_2/M regulators, such as Cdc2 and cyclin B1, in a concentration-dependent fashion (Fig. 4B). Based on these findings, eupatilin is

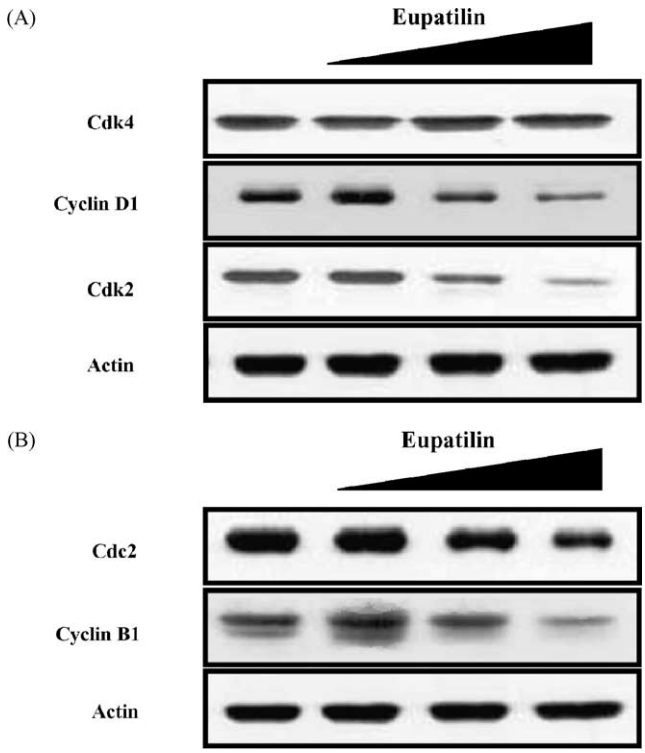


Fig. 4. Effects of eupatilin on expression of the selected cell cycle regulators of G1/S (A) and G2/M (B) transition of the cell cycle. MCF10A-*ras* cells were treated with 10, 50, or 100 μM of eupatilin for 48 h, and cellular extracts were analyzed for protein expression by Western blot analysis. Blots are representative of three independent experiments.

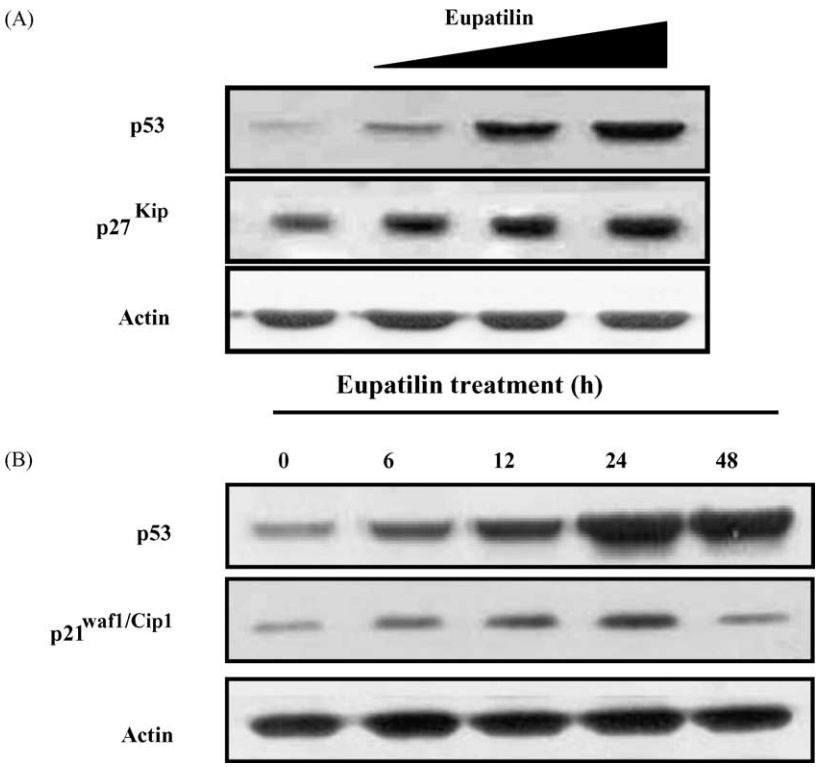


Fig. 5. Effects of eupatilin on expression of Cdk inhibitors. (A) MCF10A-*ras* cells were treated with 10, 50 or 100 μM of eupatilin for 48 h. (B) MCF10A-*ras* cells were treated with 100 μM of eupatilin for the time indicated. The protein levels of p53, $p27^{Kip1}$ and $p21^{waf1/Cip1}$ were analyzed by Western blot analysis.

likely to block the cell cycle progression through G_1 to S and G_2 to M phases.

3.4. Eupatilin upregulated p53 and $p27^{Kip1}$

To examine whether eupatilin could induce the Cdk inhibitor proteins, we investigated its effect on the expression of $p27^{Kip1}$ and $p21^{waf1/Cip1}$ proteins. The expression of $p27^{Kip1}$ was substantially increased after exposure to eupatilin for 48 h (Fig. 5A). However, the expression of $p21^{waf1/Cip1}$ was transiently increased and returned to the basal level after 48 h treatment (Fig. 5B). It has been known that the expression of $p21^{waf1/Cip1}$ is regulated via a p53-dependent or -independent mechanism [16]. The p53 tumor suppressor has been shown to be a key regulator in inducing the cell cycle arrest. The levels of p53 increased concentration dependently in MCF10A-*ras* cells treated with eupatilin (Fig. 5A). The time course study demonstrated that expression of p53 was constantly upregulated during 48 h, but expression of $p21^{waf1/Cip1}$ was transiently elevated by eupatilin treatment (Fig. 5B).

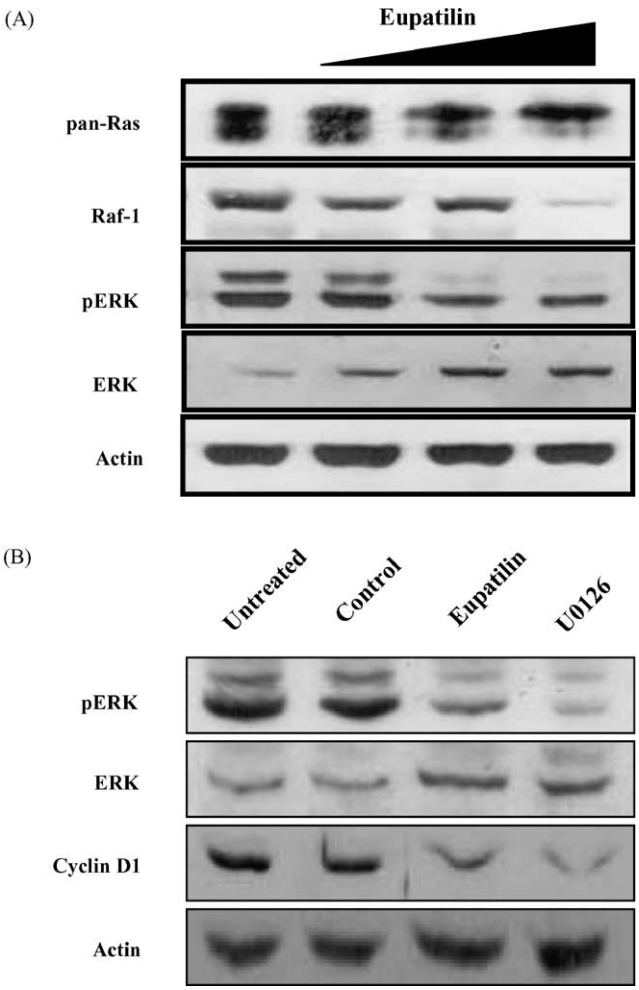


Fig. 6. Effects of eupatilin on the Ras/Raf/MAPK pathway. (A) Total cell lysates from MCF10A-*ras* cells treated with varying concentrations (10, 50, and 100 μM) of eupatilin were analyzed for pan-Ras, Raf-1, phospho-ERK and total-ERK. (B) Eupatilin (100 μM) and U0126 (10 μM) were treated to MCF10A-*ras* cells for 48 h, and their effects on cyclin D1 expression as well as phosphorylation of ERK were examined.

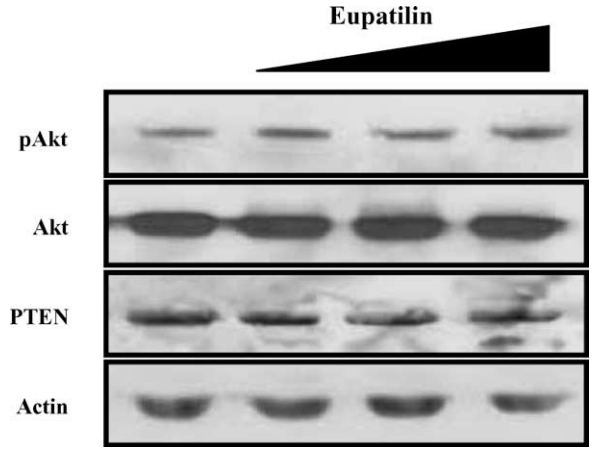


Fig. 7. Effects of eupatilin on the PI3K/Akt signaling. MCF10A-*ras* cells were treated with eupatilin as described in the legend of Fig. 6, and protein lysates were subjected to Western blot analysis of pAkt, Akt and PTEN.

3.5. Eupatilin decreased expression of cyclin D1 via the Ras/Raf/MAPK signaling pathway

To further elucidate the molecular basis for the eupatilin-induced cell cycle arrest, we measured the expression of major proteins of the Ras-signaling pathway in MCF10A-*ras* cells after eupatilin treatment for 48 h. As shown in Fig. 6A, both pan-Ras and Raf-1 protein levels decreased by eupatilin treatment. In addition, ERK activation through phosphorylation was attenuated significantly while the level of total-ERK increased slightly (Fig. 6A).

Cyclin D1 is considered to play a pivotal role in mediating the growth factor signaling responsible for cell cycle regulation [17]. To confirm whether the regulation of cyclin D1 by eupatilin in MCF10A-*ras* cells is mediated via the ERK pathway, we utilized U0126 that is an ultrapotent inhibitor of MEK1/2 responsible for activation of ERK. MCF10A-*ras* cells treated with eupatilin (100 μM) or U1026 (10 μM) for 48 h exhibited lower levels of phosphorylated ERK as well as cyclin D1 (Fig. 6B).

In another experiment, we examined whether expression of cyclin D1 could be influenced by PI3K/Akt. As shown in Fig. 7, neither phosphorylation of Akt nor expression of PTEN, an inactivator of Akt/PKB, was altered by eupatilin (100 μM) treatment.

4. Discussion

Uncontrolled cell proliferation is the hallmark of cancer, and tumor cells have typically acquired mutations in proteins that regulate the cell cycle progression in a coordinated fashion. Recently, a wide array of edible

phytochemicals have been reported to induce growth inhibition of various cancer cells by interfering with their deregulated cell cycle progression. For instance, resveratrol and curcumin inhibit the growth of Ha-*ras* transformed cell lines through modulation of the expression and/or activities of key proteins required for cell cycle regulation [18,19]. In our present study, eupatilin, an active component of *A. asiatica*, induced cell cycle arrest through down-regulation of constitutively activated Ras/Raf/ERK signaling, leading to suppression of cyclin D1 expression.

Cyclin D1 plays a critical role in the progression of mammalian cells through the G_1 phase of the cell cycle. Inappropriate overexpression of cyclin D1 is frequently observed in several types of human cancer [20]. Its promoter is one of the major targets of several growth stimulatory signals [21,22]. Thus, cyclin D1 is induced by a variety of proliferative and transforming signals such as Neu, Ras, Rac, Src, STATs, β-catenin, and activating mutants of MAPK. Among these signals, three members of the RAS family, H-, K-, and N-*ras*, are found to be activated by mutation in human tumors [23]. The *ras* gene, which is highly conserved in evolution, plays an essential role in cellular proliferation, development, and differentiation [23–26]. The first mammalian effector of Ras is the protein serine/threonine kinase Raf. Raf phosphorylates and activates MEK1 and MEK2 which, in turn, phosphorylate ERK1 and ERK2, respectively.

Recent studies have suggested functional interactions between Ras and cyclin D1 [27]. It has been suggested that transforming mutants of Ras induce the cyclin D1 promoter in human trophoblasts, mink lung epithelial, and Chinese hamster ovary fibroblasts [28–30]. Ras acts at several distinct phases of the cell cycle including early G_1 and the G_1/S or G_2/M boundary. Activation of MAPKs induces reentry into the cell cycle, implicating MAPKs as targets in the transmission of proliferative signals. Cyclin D1 promoter activity was also stimulated by overexpression of MAPKs, and transfection with plasmids encoding dominant negative MAPK antagonized MAPK-dependent induction of cyclin D1 promoter activity [30,31]. Ras can also interact directly with the catalytic subunit of PI3K. PI3K controls the activity of a number of downstream enzymes. Among them, Akt/PKB has a strong anti-apoptotic function and is an important part of the survival signal that is generated by Ras. These include increased proliferation due to induction of cell cycle regulators, such as cyclin D1 [32]. However, we observed that phosphorylation of Akt and expression of its negative regulator PTEN were not altered in MCF10A-*ras* cells treated with eupatilin, although expression of Raf and phosphorylation of ERK decreased. The MEK inhibitor U0126 and eupatilin reduced expression of cyclin D1, suggesting that eupatilin-mediated cell cycle arrest could be achieved by targeting the Raf/MEK pathway.

The Cdk inhibitor, $p27^{Kip1}$ also plays an important part in mediating growth arrest and is thought to function as a brake of the cell cycle [33,34]. Another Cdk inhibitor $p21^{waf1/Cip1}$, which is a transcriptional target of p53, is a negative regulator of cell cycle progression. $p21^{waf1/Cip1}$ is induced in various cancer cells undergoing cell cycle arrest, apoptosis, and differentiation. $p21^{waf1/Cip1}$ expression is usually controlled at the transcriptional level via a p53-dependent or -independent mechanism. We noted that eupatilin induced expression of p53 in a time- and concentration-dependent manner, which was accompanied by upregulation of $p27^{Kip1}$ and $p21^{waf1/Cip1}$.

Recently, it has been reported that cyclin D1 is a critical mediator of Ras-induced $p21^{waf1/Cip1}$ stability in *ras*-transformed cells [27]. According to this work, Ras-induced $p21^{waf1/Cip1}$ stabilization was dependent upon cyclin D1 expression. $p21^{waf1/Cip1}$ directly binds to the C8α subunit of the 20S proteasome complexes, and cyclin D1 inhibits $p21^{waf1/Cip1}$ degradation. Ras stabilizes $p21^{waf1/Cip1}$ by promoting the formation of $p21^{waf1/Cip1}$-cyclin D1 complexes that prevent $p21^{waf1/Cip1}$ association with the 20S proteasome and subsequently degradation by it [27]. Moreover, we observed that expression of $p21^{waf1/Cip1}$ paralleled that of cyclin D1 in a time course study (data not shown). Therefore, we assume that regulation of $p21^{waf1/Cip1}$ expression is influenced by cyclin D1 in MCF10A-*ras* cells.

In conclusion, eupatilin inhibited the growth of MCF10A-*ras* cells through induction of cell cycle arrest. The cytostatic effect of eupatilin appears to be associated with its down-regulation of cyclin D1 whose expression appears to be under the control of the Raf/MEK/ERK signaling pathway (Fig. 8).

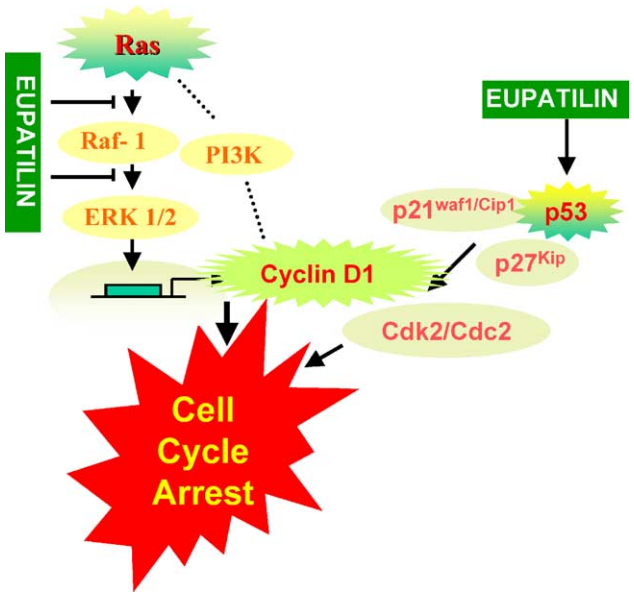


Fig. 8. (A) putative mechanism of eupatilin-induced cell cycle arrest in MCF-10A-*ras* cells. Eupatilin is proposed to inhibit expression of cyclin D1 via the Ras/Raf/MAPK signaling pathway, independently of the Akt pathway. In addition, eupatilin-induced cell cycle arrest appears to be associated with up-regulation of p53 and $p27^{Kip1}$.

Acknowledgments

This work was supported by the grant from the KISTEP, Ministry of Science and Technology, for Functional Food Research and Development.

References

[1] Wenzel U, Kuntz S, Brendel MD, Daniel H. Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells. Cancer Res 2000;60:3823–31.
[2] Pan MH, Chen WJ, Lin-Shiau SY, Ho CT, Lin JK. Tangeretin induces cell-cycle G1 arrest through inhibiting cyclin-dependent kinases 2 and 4 activities as well as elevating Cdk inhibitors p21 and p27 in human colorectal carcinoma cells. Carcinogenesis 2002; 23:1677–84.
[3] Hirano T, Abe K, Gotoh M, Oka K. Citrus flavone tangeretin inhibits leukaemic HL-60 cell growth partially through induction of apoptosis with less cytotoxicity on normal lymphocytes. Br J Cancer 1995;72:1380–8.
[4] Seo HJ, Park KK, Han SS, Chung WY, Son MW, Kim W-B, et al. Inhibitory effects of the standardized extract (DA-9601) of *A. asiatica* Nakai on phorbol ester-induced ornithine decarboxylase activity, papilloma formation, cyclooxygenase-2 expression, inducible nitric oxide synthase expression and nuclear transcription factor kappa B activation in mouse skin. Int J Cancer 2002;100:456–4562.
[5] Seo HJ, Surh Y-J. Eupatilin, a pharmacologically active flavone derived from *Artemisia* plants, induces apoptosis in human promyelocytic leukemia cells. Mutat Res 2001;496:191–8.
[6] Clair T, Miller WR, Cho-Chung YS. Prognostic significance of the expression of a ras protein with a molecular weight of 21,000 by human breast cancer. Cancer Res 1987;47:5290–3.
[7] Barbacid M. ras genes. Annu Rev Biochem 1987;56:779–827.
[8] Sewing A, Wiseman B, Lloyd AC, Land H. High-intensity Raf signal causes cell cycle arrest mediated by $p21^{Cip1}$. Mol Cell Biol 1997;17:5588–97.
[9] Mulcahy LS, Smith MR, Stacey DW. Requirement for *ras* proto-oncogene function during serum-stimulated growth of NIH 3T3 cells. Nature 1985;313:241–3.
[10] Woods D, Parry D, Cherwinski H, Bosch E, Lees E, McMahon M. Raf-induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by $p21^{Cip1}$. Mol Cell Biol 1997;17:5598–611.
[11] Schulze A, Lehmann K, Jefferies HB, McMahon M, Downward J. Analysis of the transcriptional program induced by Raf in epithelial cells. Genes Dev 2001;15:981–94.
[12] Janes PW, Daly RJ, deFazio A, Sutherland RL. Activation of the Ras signalling pathway in human breast cancer cells overexpressing erbB-2. Oncogene 1994;9:3601–8.
[13] Bos JL. ras oncogenes in human cancer: a review. Cancer Res 1989;49:4682–9.
[14] Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev 1998;12:3499–511.
[15] Radu A, Neubauer V, Akagi T, Hanafusa H, Georgescu MM. PTEN induces cell cycle arrest by decreasing the level and nuclear localization of cyclin D1. Mol Cell Biol 2003;23:6139–49.
[16] Parker SB, Eichele G, Zhang P, Rawls A, Sands AT, Bradley A, et al. p53-independent expression of $p21^{Cip1}$ in muscle and other terminally differentiating cells. Science 1995;267:1024–7.
[17] Kerkhoff E, Rapp UR. Cell cycle targets of Ras/Raf signalling. Oncogene 1998;17:1457–62.
[18] Kim MS, Kang HJ, Moon A. Inhibition of invasion and induction of apoptosis by curcumin in H-*ras*-transformed MCF10A human breast epithelial cells. Arch Pharm Res 2001;24:349–54.
[19] Lin HY, Shih A, Davis FB, Tang HY, Martino LJ, Bennett JA, et al. Resveratrol induced serine phosphorylation of p53 causes apoptosis in a mutant p53 prostate cancer cell line. J Urol 2002;168:748–55.
[20] Jiang W, Zhang YJ, Kahn SM, Hollstein MC, Santella RM, Lu SH, et al. Altered expression of the cyclin D1 and retinoblastoma genes in human esophageal cancer. Proc Natl Acad Sci USA 1993;90:9026–30.
[21] Pestell RG, Albanese C, Watanabe G, Johnson J, Eklund N, Lastowiecki P, et al. Epidermal growth factor and c-Jun act via a common DNA regulatory element to stimulate transcription of the ovine P-450 cholesterol side chain cleavage (CYP11A1) promoter. J Biol Chem 1995;270:18301–8.
[22] Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, et al. The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. Proc Natl Acad Sci USA 1999;96:5522–7.
[23] Lowy DR, Willumsen BM. Function and regulation of ras. Annu Rev Biochem 1993;62:851–91.
[24] Boguski MS, McCormick F. Proteins regulating Ras and its relatives. Nature 1993;366:643–54.
[25] Feig LA. The many roads that lead to Ras. Science 1993;260:767–8.
[26] McCormick F. Signal transduction. How receptors turn Ras on. Nature 1993;363:15–6.
[27] Coleman ML, Marshall CJ, Olson MF. Ras promotes $p21^{waf1/Cip1}$ protein stability via a cyclin D1-imposed block in proteasome-mediated degradation. EMBO J 2003;22:2036–46.
[28] Watanabe G, Lee RJ, Albanese C, Rainey WE, Batlle D, Pestell RG. Angiotensin II activation of cyclin D1-dependent kinase activity. J Biol Chem 1996;271:22570–7.
[29] Shambaugh III GE, Lee RJ, Watanabe G, Erfurth F, Karnezis AN, Koch AE, et al. Reduced cyclin D1 expression in the cerebella of nutritionally deprived rats correlates with developmental delay and decreased cellular DNA synthesis. J Neuropathol Exp Neurol 1996;55:1009–20.
[30] Albanese C, Johnson J, Watanabe G, Eklund N, Vu D, Arnold A, et al. Transforming $p21^{ras}$ mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. J Biol Chem 1995;270:23589–97.
[31] Weber JD, Raben DM, Phillips PJ, Baldassare JJ. Sustained activation of extracellular-signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in G1 phase. Biochem J 1997;326(Pt 1):61–8.
[32] Downward J. Targeting Ras signalling pathways in cancer therapy. Nat Rev Cancer 2003;3:11–22.
[33] Sherr CJ. Cancer cell cycles. Science 1996;274:1672–7.
[34] Fero ML, Rivkin M, Tasch M, Porter P, Carow CE, Firpo E, et al. A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in $p27^{Kip1}$-deficient mice. Cell 1996;85:733–44.